ION CHANNEL MODULATORS

RELATED APPLICATIONS

[0001] The present application is a Continuation of co-pending PCT Application No. PCT/GB02/02919 filed on June 21, 2002, which in turn, claims priority from Great Britain Application Serial No. 0115363.4, filed June 22, 2001. Applicants claim the benefits of 35 U.S.C. §120 as to the PCT application and priority under 35 U.S.C. §119 as to said Spanish application, and the entire disclosures of both applications are incorporated herein by reference in their entireties.

[0002] The present invention relates to ion channel modulators. In particular, the invention relates to ion channel modulators that are derived from haematophagous arthropods. The invention also relates to the use of ion channel modulators from haematophagous arthropods in the treatment and prevention of certain diseases and conditions in mammals, including humans.

[0003] Ion channels are proteins which allow ions to cross the lipid bilayer of cell membranes. They exert control over the movement of ions by being either open or closed. Ion channel proteins can be divided into two groups. The first group of ion channel proteins form narrow hydrophilic pores across the membrane, allowing the passive movement of small inorganic ions. The second group of ion channel proteins can be coupled to a source of energy to promote active transport of ions across the cell membrane. Through a combination of passive and active transport using ion channels, cells can generate ionic concentration differences across the lipid bilayer of cell membranes.

[0004] The electrical gradient thus generated can be used by cells in their signalling and control systems. Ion channels show selectivity with respect to the ions to which they are permeable and respond to different opening and closing stimuli (eg ligand gated or voltage gated channels). Modulators of ion channels can be divided into blocking agents

which can only close channels and modulators which can either open or close them (Hille, 1992)

[0005] The present invention is particularly concerned with the modulation of ion channels in vascular smooth muscle cells and cardiac muscle cells. In such cells, propagation of an action potential over the membrane surface of the cell is associated with contraction of the cell. This effect is usually achieved by a small change in the resting membrane potential triggering the opening of a voltage gated ion channel which then permits the influx of large quantities of positively charged ions with a consequent change in the electrostatic conformation of the cell. Typically, the small change in the resting membrane potential is achieved through active transport of Na^+ and K^+ ions by the Na^+- K^+ pump. This pump depends on an energy source to drive the process and this is provided by the catalysis of ATP by the enzyme Na,K-ATPase.

[0006] The contraction of smooth muscle, including cardiac smooth muscle, is dependent on a transient increase in cytoplasmic Ca^{++} concentration, such ions coming from two sources: influx from the extracellular compartment by opening of inward Ca^{++} channels and release of intracellular Ca^{++} ions from the cisternae of the sarcoplasmic reticulum. Calcium channel blocking agents that inhibit the influx of extracellular Ca^{++} ions will therefore tend to relax most smooth muscle cells including cardiac cells. The force of contraction of cardiac smooth muscle is termed the inotropic state and, therefore, agents which relax the resting state of cardiac smooth muscle cells, such as most calcium channel blockers, are negatively inotropic. Conversely agents that increase the force of contraction of cardiac smooth muscle are positively inotropic.

[0007] Calcium channel blockers are used therapeutically to relax vascular smooth muscle thereby increasing the diameter of blood vessels ('vasodilation') in order to lower blood pressure or increase regional blood flow (eg via the coronary arteries). The coincidental lowering of the force of cardiac smooth muscle contraction is an inherent disadvantage of most calcium channel blocking agents and precludes their use in conditions in which the functioning of the heart is already compromised. Such conditions

include congestive heart failure, cardiomyopathies, septicaemia and following myocardial infarction. (Alberts et al, 1998; Camm, 1996a; Hume et al, 1998; Akera et al, 1998).

[0008] There is therefore a need for agents which possess the desirable qualities of vasodilators but which are not negatively inotropic. Preferably, such agents would be positively inotropic.

[0009] In situations in which it is desired to increase cardiac output, drugs having a positive inotropic effect may be used. Positively inotropic drugs such as digoxin frequently work through inhibition of sodium-potassium ATPase (Camm, 1996b). However, currently known positively inotropic drugs, which include digoxin, dopamine, isopraline and phopshodiesterase inhibitors such as enoximone and milrinone, often have other, undesirable, effects on the heart such as the induction of arrhythmia.

[0010] There is therefore a need for agents which have a positive intropic effect on the heart without inducing arrythmias.

[0011] Given the importance of ion channels in disease, there is thus a need for the identification of novel ion channel modulators with improved properties. More specifically, in view of the widespread incidence of cardiovascular disease and the disadvantages of drugs that are currently available, there remains a need for improved ion channel modulators in the treatment of cardiovascular disease. In particular, there remains a great need for ion channel modulators with properties which are useful in the treatment of cardiovascular disease, such as those that induce vasodilation and that are positively inotropic. In addition, there is a need for ion channel modulators which can induce a positive inotropic effect without having adverse effects, such as inducing arrhythmia.

[0012] It has now been discovered that suitable ion channel modulators can be isolated from haematophagous arthropods.

SUMMARY OF THE INVENTION

[0013] According to a first aspect of the present invention, there is provided an ion channel modulator molecule (ICMM), derived from an haematophagous arthropod, or a functional equivalent thereof.

[0014] By "ion channel modulator molecule" is meant any molecule that modulates the activity of an ion channel. Preferably, the ICMMs are proteins or peptides. However, they may also be non-peptidic derivatives. Preferably, non-peptidic derivatives are small organic molecules.

[0015] By "ion channel" is meant any transmembrane protein or transmembrane protein complex present in the cell that allows the movement of particular ions from one side of a cell membrane to the other.

[0016] The ICMMs or functional equivalents of the present invention modulate the activity of ion channels that allow the movement of ions across the cell membrane by either active or passive transport. Examples of ion channels that allow the movement of ions by active transport include calcium ATPase and sodium-potassium ATPase. Examples of ion channels that allow movement of ions by passive transport include calcium channels, sodium channels and potassium channels. These channels tend to be of particular relevance to disease, such as cardiac disease.

[0017] The ICMMs or functional equivalents of the invention may modulate more than one ion channel or class of ion channel. Such modulators may allow the simultaneous modulation of ion channels of different classes. For example, ICMMs may permit

vasodilation by means of blockade of one or more calcium channels whilst also causing positive inotropism by means of inhibition of the sodium-potassium ATPase channel.

[0018] The ICMMs or functional equivalents of the present invention may modulate the activity of ion channels by either inhibiting or promoting the activity of ion channels. Preferably, the ICMMs or functional equivalents of the invention inhibit the activity of ion channels. One example of an ion channel that the ICMMs or functional equivalents of the present invention may inhibit is the sodium-potassium ATPase ion channel. Preferably, ICMMs or functional equivalents of the invention inhibit the activity of ion channels by binding to them.

[0019] The ICMMs or functional equivalents may be vasodilators. Vasodilation may be promoted by the ICMMs functional equivalents of the invention, for example, through blockade of calcium channels or by nitric oxide donation. The ICMMs or functional equivalents may be vasodilators of coronary vessels, peripheral vessels or both types of vessel. Preferably, the ICMMs, or functional equivalents of the invention act as vasodilators of both coronary vessels and peripheral vessels.

[0020] The ICMMs or functional equivalents of the invention preferably do not induce a negative inotropic effect in cardiac smooth muscle. Preferably, the ICMMs or functional equivalents induce a positive inotropic effect.

[0021] The ICMMs or functional equivalents of the invention may function to prolong the action potential of muscle cells. Preferably, the ICMMs or functional equivalents of the invention prolong the action potential of cardiomyocyte cells. This property may be important in prevention or treatment of arrhythmias such as are sometimes associated with the use of other positively inotropic drugs.

[0022] The ICMMs or functional equivalents of the present invention are derived from haematophagous arthropods. The term "haematophagous arthropod" includes all

arthropods that take a blood meal from a suitable host, and includes insects, ticks, lice, fleas and mites.

[0023] Preferably, the ICMMs or functional equivalents of the present invention are derived from horseflies of the *Tabanidae* family. More preferably, they are derived from horseflies of the *Hybomitra, Heptatoma, Chrysops, Haematopota* and *Tabanus* genera. Most preferably, they are derived from the horsefly *Hybomitra bimaculata.*

[0024] The term "functional equivalent" is used herein to describe variants, derivatives or fragments of ICMMs of the invention that retain the ability to modulate ion channels. Functional equivalents of the ICMMs of the present invention thus include natural biological variants (e.g. allelic variants or geographical variants within the species from which the ICMMs are derived).

[0025] Variants of the proteinaceous ICMMs of the invention also include, for example, mutants containing amino acid substitutions, insertions or deletions from the wild type sequence. Variants with improved function from that of the wild type sequence may also be designed through the systematic or directed mutation of specific residues in the protein sequence. Improvements in function that may be desired will include greater specificity for the target ion channel or greater affinity for the target ion channel.

[0026] The term "functional equivalent" also refers to molecules that are structurally similar to the proteinaceous ICMMs of the present invention or that contain similar or identical tertiary structure, particularly in the environment of the active site. Such functional equivalents may thus be derived from natural proteinaceous ICMMs or they may be prepared synthetically or using techniques of genetic engineering. In particular, synthetic molecules that are designed to mimic the tertiary structure or active site of the natural proteinaceous ICMMs of the invention are considered to be functional equivalents.

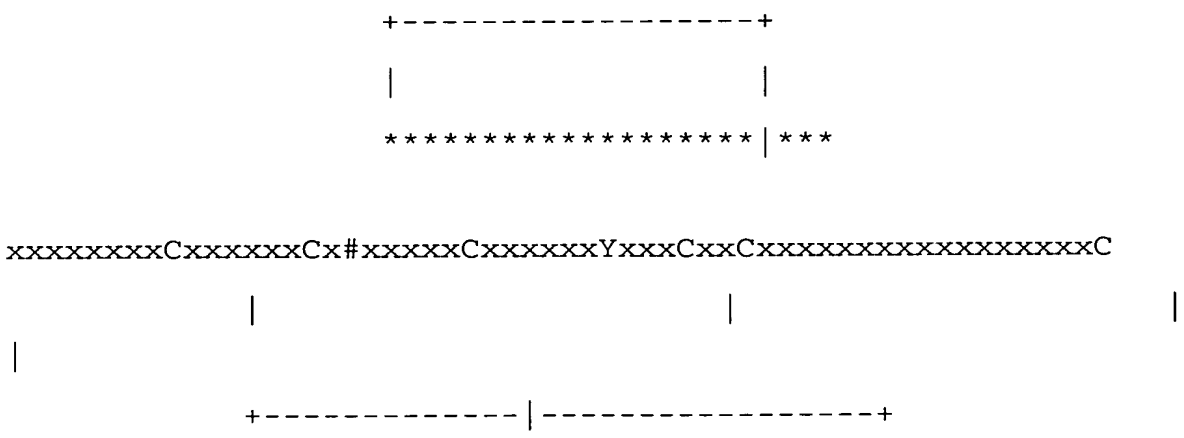
[0027] The term "functional equivalent" also includes fragments of the proteinaceous ICMMs of the present invention, fragments of variants of the proteinaceous ICMMs and fragments of structurally similar molecules, provided such fragments retain the ability to modulate ion channels. Preferably, protein fragments according to the invention comprise the amino acid sequence psggrrs. This amino acid sequence may be the active site of proteinaceous ICMMs, although the Applicant does not wish to be bound by this theory.

[0028] The term "functional equivalent" also refers to homologues of the proteinaceous ICMMs. By "homologue" is meant a protein exhibiting a high degree of similarity or identity to the amino acid sequence of a natural proteinaceous ICMM. By "similarity" is meant that, at any particular position in the aligned sequences, the amino acid residue is of a similar type between the sequences. By "identity" is meant that at any particular position in the aligned sequences, the amino acid residue is identical between the sequences.

[0029] Preferably, homologues possess greater than 50% identity with the sequence of the natural protein. More preferably, homologues according to the invention show greater than 60%, 70%, 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity with the sequence of the natural protein, as aligned using, for example, the GCG suite of programs (Wisconsin Package Version, 10.1, Genetics Computer Group (GCG), Madison, Wisc.) or the ExPASy (ExpertProtein Analysis System) proteomics server of the Swiss Insitute of Bioinformatics. Tools such as PROSITE (http://expasy.hcuge.ch/sprot/prosite.html), PRINTS http://iupab.leeds.ac.uk/bmb5dp/prints.html), Profiles (http://ulrec3.unil.ch/software/ PFSCAN_form.html), Pfam (http://www.sanger.ac.uk/software/pfam), Identify (http://dna.stanford.edu/identify/) and Blocks (http://www.blocks.fhcrc.org) databases may also be used to identify homologues, as well as hidden Markov models (HMMs; preferably profile HMMs). Such homologues may include proteins in which one or more of the amino acid residues are substituted with another amino acid residue and such substituted amino acid residue may or may not be a naturally occurring amino acid.

[0030] The term "functional equivalent" also includes derivatives of the present invention. Such derivatives may include one or more additional peptides or polypeptides fused at the amino- or carboxy- terminus of the proteinaceous ICMMs, fragments or homologues. The purpose of the additional peptide or polypeptide may be to aid the detection, expression, separation or purification of the protein or it may endow the protein with additional properties, as desired. Examples of useful fusion partners include beta-galactosidase, glutathione-S-transferase, luciferase, a polyhistidine tag, a T7 polymerase fragment and a secretion signal peptide. Such derivatives may be prepared by fusing the peptides genetically or chemically.

[0031] The proteinaceous ICMMs or functional equivalents of the present invention may show some homology to Kazal type proteins. The Kazal family of proteins includes a variety of protease inhibitors including pancreatic secretory trypsin inhibitor (Greene and Giordano, 1969), avian ovomucoid (Laskowski *et al*, 1987), acrosin inhibitor (Williamson *et al*, 1984) and elastase inhibitor (Tschesche *et al*. 1987). The basic structure of a Kazal-type inhibitor is shown in the following schematic representation:



Kazal inhibitors contain between 1 and 9 Kazal-type inhibitor repeats.

The Applicant has demonstrated that at least one proteinaceous ICMM shows homology with Kazal type proteins and believes that other Kazal type proteins may act as ICMMs.

[0032] A further embodiment of the invention therefore provides the use of a Kazal type protein, or functional equivalent thereof as an ICMM. Functional equivalents of Kazal type proteins include fragments and variants of Kazal type proteins, providing that said fragments and variants retain ion channel modulatory activity. In particular, the term functional equivalent is used to describe variants of Kazal proteins obtained by mutation or trunction of a Kazal type protein.

[0033] According to a preferred embodiment of the invention, the ICMM comprises the amino acid sequence set out in Figure 9a, or a functional equivalent thereof. The ICMM comprising this sequence is referred to herein as EV048. Functional equivalents of EV048 include variants, fragments, homologues or derivatives as defined above, providing that said variants, fragments homologues or derivatives retain activity as ion channel modulators. Amino acids 1 to 20 of the EV048 amino acid sequence set out in Figure 9a form a signal sequence. Functional equivalents of EV048 include fragments of the amino acid sequence set out in Figure 9a which do not contain the signal sequence.

[0034] Preferably, variants, fragments, homologues or derivatives of EV048 should retain structural homology or conservation of the putative active site of EV048. This active site may reside in the 7 amino acid sequence psggrrs between the third and fourth cysteine molecules in the sequence, although the Applicant does not wish to be bound by this theory.

[0035] According to a further embodiment of the invention, there is provided the use of a peptide or polypeptide comprising the sequence psggrrs as an ion channel modulator, as defined above.

[0036] The invention also provides a screening method for the identification of variants of EV048 which have enhanced ion modulatory properties compared to EV048. According to this method, variants of EV048, preferably mutants, are created and these mutants are tested for the ability to modulate ion channel activity. In particular, such mutants are tested for their ability to cause vasodilation and positive inotropism.

[0037] The ICMMs or functional equivalents of the invention may be prepared in recombinant form by expression in a host cell. Suitable expression methods are well known to those of skill in the art and many are described in detail by Sambrook *et al* (2000) and Fernandez & Hoeffler (1998).

[0038] The proteins or functional equivalents of the present invention can also be prepared using conventional techniques of protein chemistry, for example by chemical synthesis.

[0039] According to a second aspect of the invention, there is provided a nucleic acid molecule comprising a nucleotide sequence encoding an ICMM or functional equivalent thereof, according to the first aspect of the invention. Such nucleic acid molecules include single- or double-stranded DNA, cDNA and RNA, as well as synthetic nucleic acid species. Preferably, the nucleic acid molecules are DNA or cDNA molecules.

[0040] The invention also includes cloning and expression vectors incorporating the nucleic acid molecules of the second aspect of the invention. Such expression vectors may additionally incorporate the appropriate transcriptional and translational control sequences, for example enhancer elements, promoter-operator regions, termination stop sequences, mRNA stability sequences, start and stop codons or ribosomal binding sites, linked in frame with the nucleic acid molecules of the second aspect of the invention.

[0041] Additionally, it may be convenient to cause a recombinant protein to be secreted from certain hosts. Accordingly, further components of such vectors may include nucleic acid sequences encoding secretion, signalling and/or processing sequences.

[0042] Vectors according to the invention include plasmids and viruses (including both bacteriophage and eukaryotic viruses), as well as other linear or circular DNA carriers, such as those employing transposable elements or homologous recombination technology. Many such vectors and expression systems are known and documented in the art (see, for example, Fernandez & Hoeffler, 1998). Particularly suitable viral vectors include baculovirus-, adenovirus- and vaccinia virus- based vectors.

[0043] Suitable hosts for recombinant expression include commonly used prokaryotic species, such as *E. coli*, or eukaryotic yeasts that can be made to express high levels of recombinant proteins and that can easily be grown in large quantities. Mammalian cell lines grown *in vitro* are also suitable, particularly when using virus-derived expression systems. Another suitable expression system is the baculovirus expression system, that involves the use of insect cells as hosts. An expression system may also constitute host cells that have the appropriate encoding nucleic acid molecules incorporated into their genome. Proteins, or protein fragments may also be expressed *in vivo*, for example in insect larvae or in mammalian tissues.

[0044] A variety of techniques may be used to introduce the vectors according to the present invention into prokaryotic or eukaryotic host cells. Suitable transformation or transfection techniques are well described in the literature (see, for example, Sambrook *et al*, 2000; Ausubel *et al*, 1991; Spector, Goldman & Leinwald, 1998). In eukaryotic cells, expression systems may either be transient (e.g. episomal) or permanent (such as by chromosomal integration) according to the needs of the system.

[0045] The invention also includes transformed or transfected prokaryotic or eukaryotic host cells containing a nucleic acid molecule as defined above.

[0046] A further aspect of the invention provides a method for preparing an ICMM or a functional equivalent thereof as defined above, which comprises culturing a host cell containing a nucleic acid according to the invention under conditions whereby said protein is expressed and recovering said protein thus produced.

[0047] A further aspect of the invention provides a method for isolating an ICMM or functional equivalent thereof, as defined above comprising the steps of: preparing an extract from a haematophagous arthropod as defined previously; separating said extract into fractions containing different proteins; testing said fractions for the ability to modulate, preferably inhibit, ion channel activity; and isolating said ICMM or functional equivalent thereof, from a fraction(s) that possesses the ability to modulate, preferably inhibit, ion channel activity.

[0048] Preferably, said extract is a salivary gland extract. Depending on the arthropod species the preparation of such salivary gland extracts may take place at any one of several suitable points in the feeding cycle. In the case of *Hybomitra bimaculata,* the extract is preferably prepared from the salivary glands of adult females since this is the only haematophagous instar.

[0049] In a particularly preferred embodiment of the invention, an ICMM or functional equivalent thereof may be obtained by a method comprising the steps of:

a) preparing a salivary gland extract from a haematophagous arthropod;

b) separating said extract into fractions containing proteins;

c) testing said fractions for the ability to modulate the activity of an ion channel; and

d) isolating said ICMM or functional equivalent thereof from a fraction(s) that possesses the ability to modulate ion channel activity.

[0050] Preferably, the haematophagous arthropod used in the above method is a horsefly of the *Tabinadae* family. Preferably, the ICMMs or functional equivalents, including EV048 and homologues thereof, are derived from horseflies of the *Hybomitra, Heptatoma, Chrysops, Haematopota* and *Tabanus* genera using the above-described method, more preferably, from *Hybomitra bimaculata*.

[0051] Suitable methods of separating haematophagous arthropod extracts into fractions containing purified proteins will be apparent to those skilled in the art. Preferably, the extract is separated into fractions of may be carried out using a chromatographic procedure, such as fast phase liquid chromatography (FPLC), high-performance liquid chromatography (HPLC), ion exchange chromatography, affinity chromatography, gel filtration or reverse phase HPLC.

[0052] Testing of the fractions for their ability to modulate ion channel activity, in particular to cause vasodilation and/or positive inotropism and/or lengthen action potential, can also be carried out by one of several methods known to those skilled in the art. The methods used for assessing whether modulation of ion channels has been effected may vary depending on the ion channel under consideration. For example, in the case of the sodium-potassium ATPase, the activity of ATPase on varying concentrations of ATP in the presence and absence of fractions containing putative ICMMs may be assessed. In the case of inotropism the effect of fractions on whole cell patch clamping in isolated cardiomyocytes or the effect on left ventricular output in an isolated perfused Langendorf rat heart may be assessed. The effect on lengthening of action potential may be assessed by whole cell patch clamping in isolated cardiomyocytes. Vasodilation may be assessed by the effect of the fractions containing putative ICMMs on pre-contracted rat femoral artery rings or by assessing the effect of fractions on coronary blood flow in an isolated Langendorf heart.

[0053] Following identification of a fraction that possesses the ability to modulate ion channel activity, an ICMM or functional equivalent thereof may be isolated by any

suitable procedures, including procedures such as SDS-polyacrylamide gel electrophoresis or two dimensional gel electrophoresis.

[0054] The present invention also includes an ICMM or functional equivalent thereof obtainable by any one of the methods described above. Preferably, the ICMM or functional equivalent thereof modulates the activity of a sodium channel, a potassium channel, a calcium channel and/or a sodium-potassium ATPase. The ICMM or functional equivalent thereof may modulate the activity of more than one ion channel. The ICMM or functional equivalent thereof exhibits the preferred ion channel modulatory properties listed above.

[0055] The method set out above may further comprise isolating and sequencing a gene encoding an ICMM, or functional equivalent thereof obtained using any of the methods set out above. For example, an isolated and purified ICMM may be subjected to a step of amino acid sequencing, followed by screening of a salivary gland gene library, for example using the polymerase chain reaction to isolate a gene encoding the ICMM. One example of a suitable procedure is by screening a cDNA library, optionally a cDNA expression library. Certain expression libraries can be designed to generate tagged arthropod proteins, so facilitating their analysis and purification. Suitable procedures for the preparation and isolation of parasite proteins can be found, for example, in co-owned patent applications PCT/GB97/01372 and PCT/GB98/03397. A variety of suitable procedures for isolating a gene encoding an ICMM according to the invention will be known to the skilled reader.

[0056] In a particularly preferred embodiment of the invention, a gene encoding an ICMM or functional equivalent thereof may be obtained by a method comprising performing the steps outlined in detail above to isolate the ICMM or functional equivalent thereof, and additionally performing the steps of:

e) obtaining the N-terminal sequence of said isolated ICMM or functional equivalent thereof;

f) designing a degenerate oligonucleotide; and

g) using said degenerate oligonucleotide to screen a salivary gland gene library to isolate a gene encoding the ICMM or functional equivalent thereof.

[0057] Once the ICMM or functional equivalent thereof has been isolated, sequencing of the N-terminus of the protein may be carried out by any suitable method, as will be apparent to those skilled in the art. Following the determination of the N-terminal sequence of the ICMM, or functional equivalent thereof, a skilled person will readily be able to design one or more degenerate oligonucleotide probes or degenerate PCR primers which could encode this peptide sequence. These primers may then be used to screen a salivary gland gene library, for example by hybridisation or by PCR. Preferably, such a library is a cDNA gene library.

[0058] According to a further aspect of the invention there is provided a composition comprising an ICMM or functional equivalent, or a nucleic acid comprising a nucleotide sequence encoding an ICMM or a functional equivalent according to the invention in conjunction with a pharmaceutically acceptable carrier.

[0059] Pharmaceutically-acceptable carriers include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolised molecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplets or liposomes) and inactive virus particles. Such carriers are well known to those of skill in the art.

[0060] In certain circumstances, such a composition may be used as a vaccine, and may thus optionally comprise an immunostimulating agent, for instance an adjuvant of the type referred to below. According to a further aspect of the invention, there is provided a process for the formulation of a vaccine composition comprising bringing an ICMM or

functional equivalent, or a nucleic acid comprising a nucleotide sequence encoding an ICMM or a functional equivalent according to the invention into association with a pharmaceutically-acceptable carrier, optionally with an adjuvant. Suitable adjuvants are well-known in the art and include oil-in-water emulsion formulations, saponin adjuvants, Complete Freund's Adjuvant (CFA), Incomplete Freund's Adjuvant (IFA) and other substances that may act as immunostimulating agents to enhance the effectiveness of the vaccine composition.

[0061] According to a further aspect, the present invention provides for the use of an ICMM or functional equivalent thereof in therapy.

[0062] The invention also provides a method of treating an animal suffering from a disease or condition caused by a fault in ion channel activity, comprising administering to said animal an ICMM or functional equivalent thereof, a nucleic acid comprising a nucleotide sequence encoding an ICMM or a functional equivalent thereof or a pharmaceutical composition according to the invention in a therapeutically effective amount. Preferably, said animal is a mammal, more preferably a human.

[0063] Examples of conditions suitable for treatment using the ICMMs or functional equivalents of the invention include cardiac conditions such as coronary insufficiency leading to angina, congestive cardiac failure and cardiac arrhythmias; peripheral vascular disease such as cerebro-vascular insufficiency, intermittent claudication and Buerger's disease; vasospastic disorders such as Raynaud's disease, cerebral or coronary vasospasm; reperfusion following stroke and myocardial infarction; shock including septic shock, haemorrhagic shock and cardiogenic shock; hypertension; to assist in circulatory support during and following cardio-pulmonary by-pass or angioplasty procedures.

[0064] The invention also includes the use of an ICMM, variant or functional equivalent thereof as a diagnostic tool. In particular, the invention includes the use of an ICMM,

variant or functional equivalent thereof in the diagnosis of abnormalities of the cardiovascular system. Methods of diagnosis using an ICMM, variant or functional equivalent thereof will be well known to those skilled in the art.

[0065] The present invention also includes the use of an ICMM, variant or functional equivalent thereof, as a tool in the study of ion channel modulation and the effects of ion channel modulation, including vasodilation and inotropism.

[0066] Various aspects and embodiments of the present invention will now be described in more detail by way of example, with particular reference to ICMMs isolated from horseflies and especially from *H. bimaculata*. It will be appreciated that modification of detail may be made without departing from the scope of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0067] Figure 1: Results of HPLC fractionation of crude Hybomitra bimaculata salivary gland extract. In the first purification, SGE was applied to a Vydac C-4, 250 x 4.6 mm ID, 5μm particle size column, UV monitored at 210 nm and 220 nm. A gradient of 10-100 % ACN with 0.1% TFA, flow rate 1 ml/min and with 1% ACN/min increments was used (Figure 1A). The active fraction from the first purification was further purified using a Beckman Ultrasphere C-18, 250 x 4.6mm ID, 5μm particle size column and a gradient of 10-40% ACN with 0.1% TFA, flow rate 1 ml/min with 0.5% ACN/min increments, and monitored at 210 and 220 nm (Figure 1B). For the third purification, a Vydac C 18, 250 x 4.6 mm ID, 5μm particle size column was used under the same conditions as for the second purification (Figure 1C).

[0068] Figure 2: *In vitro* effect of salivary gland extract (SGE) from *Hybomitra bimaculata* on the cardiac Na,K-ATPase activity. The SGE-quantity is expressed as amount of applied proteins from the extract.

[0069] Figure 3: In vitro effect of salivary gland extract (SGE) of *Hybomitra bimaculata* on kinetic parameters of Na,K-ATPase from rat heart. The SGE-quantity is expressed as amount of applied proteins from the extract. The data represent means $\pm$ SEM of 3 estimations, $p<0.05$.

[0070] Figure 4: Vasodilating activity of *Hybomitra bimaculata* salivary gland extract on isolated rat femoral arterial rings with and without endothelium.

[0071] Figure 5: Vasodilating activity of protein HPLC fractions (retention time 10-28 minutes) of *Hybomitra bimaculata* salivary gland extracts on rat arterial rings without endothelium.

[0072] Figure 6: Effect of 100µL *Hybomitra bimaculata* salivary gland extract on coronary blood flow in the isolated perfused rat heart.

[0073] Figure 7: Reverse phase HPLC of crude salivary gland extract of *Hybomitra bimaculata*. The peaks containing vasodilator activity are indicated with retention times.

[0074] Figure 8: N-terminal amino acid sequence of *Hybomitra bimaculata* salivary gland product EV048. Tentatively assigned residues after position 41 are indicated with a question mark.

[0075] Figure 9: Primary structure of *Hybomitra bimaculata* peptide EV048 (Figure 9a). Signal sequence underlined, cysteine residues shown in bold type, stop codon indicated by an asterisk. Alignment of peptide EV048 with Pfam consensus sequence for Kazal type proteins (Figure 9b).

[0076] Figure 10: Pfam alignment of Hybomitra bimaculata peptide Ev049 with Kazal type proteins. Gaps in the alignment are indicated by dashes and dots. Residues in lower case are outstandingly different from the overall consensus. THBI_RHOPR/6-48 and 57-101, Rhodnius prolixus thrombin inhibitor domain 1+2; IELA_ANESU/4-48, Anemonia sulcata inhibitor of elastase; AGRI, agrin; IAC, acrosin inhibitor; IOVO, ovomucoid inhibitor; QR1, quail retinal 1; SC1, secreted calcium binding 1 matric glycoprotein; SPRC, secreted protein acdic and rich in cysteine also called osteonedinand basement membrane protein 40 (bm40).

[0077] Figure 11: Coomassie blue stained NuPAGE 4-12% Bis-Tris gel showing purified peptide EV048. Lane (1) marker, (2-5) fractions h-k which elute at about 0.15M NaCl from a SP sepharose column. Marker sizes (kDa) indicated on left.

[0078] Figure 12: Effect of EV048 on isolated rat cardiomyocytes. Results of three experiments.

[0079] Figure 13: Effect of EV048 on coronary blood flow in isolated perfused rat heart. Figures 13a and 13b show the results of two separate experiments.

Examples

Materials and Methods

Horse fly collection

[0080] Horse flies were collected during the summer of 1999 in selected sites of south-western and western Slovakia using Manitoba traps. The effectiveness of the traps was improved by application of CO_2. Collections were performed during optimal weather conditions (sunny days, temperature 24 – 28°C, no wind) from May until the end of August. The collecting day started at 9:00 a.m. and finished at 5:00 p.m. Approximately 100-150 female horse flies per trap were collected each day of trapping, resulting in a total of 5,394 specimens. Horse flies were transported to the laboratory alive and then immediately processed to identify and isolate those of the species *Hybomitra bimaculata*.

Salivary gland sample preparation and purification

[0081] Prior to the dissection of salivary glands, horse-flies were immobilized for a few minutes by placing them at 4 °C. The salivary glands were dissected under a microscope and transferred to Eppendorf vials with cooled PBS buffer (0.01M phosphate buffer and 0.15M NaCl, pH 7.2). Samples were heated in a 80 °C water bath for 5 min, homogenized and centrifuged at 2,500 g for 10 min. The supernatant (referred to as crude salivary gland extract, SGE) was collected and stored at -70°C or immediately filtered through a Millex-LG syringe driven filter unit (0.20 μm, 4 mm) and processed by HPLC.

Reverse phase (RP-) HPLC

[0082] For purification and identification of vasoactive compounds, SGE of *Hybomitra bimaculata* horse flies was used. The SGE samples were diluted in 500 μl of 10% acetonitrile (ACN) with 0.1% trifluoroacetic acid (TFA) (buffer A) and loaded onto a Beckman Instruments 126/168 DAD HPLC system. In the first purification, SGE was applied to a Vydac C-4, 250 x 4.6 mm ID, 5μm particle size column, UV monitored at 210 nm and 220 nm. A gradient of 10-100 % ACN with 0.1% TFA, flow rate 1 ml/min and with 1% ACN/min increments was used (Figure 1A). The active fraction from the first purification was further purified using a Beckman Ultrasphere C-18, 250 x 4.6mm ID, 5μm particle size column and a gradient of 10-40% ACN with 0.1% TFA, flow rate 1 ml/min with 0.5% ACN/min increments, and monitored at 210 and 220 nm (Figure 1B). For the third purification, a Vydac C 18, 250 x 4.6 mm ID, 5μm particle size column was used under the same conditions as for the second purification (Figure 1C). Fractions were collected and dried in a Savant Instruments Speed-Vac.

Protein sequence analysis

[0083] Protein sequence analysis was performed by N-terminal Edman degradation using an automated sequencer (Model 494 Applied Biosystems) by Eurosequence (Groningen, the Netherlands). Reagents, chemicals and materials were obtained from Applied Biosystems (Warrington, U.K and Foster City, CA, U.S.A).

Construction of H. bimaculata *cDNA library*

[0084] One hundred pairs of *H. bimaculata* salivary glands were excised as described above and placed in 1ml RNA*later*™ (Ambion) (in place of PBS) and stored at -20°C. mRNA was isolated using the FastTrack™ 2.0 mRNA isolation kit (Invitrogen) and cDNA was synthesised using a Stratagene cDNA synthesis kit (Cat # 200401-5). After fractionation into large and small cDNAs on a Sepharose CL-2B column, the ethanol precipitated cDNA pellets were each resuspended in 3.5µl ddH_2O. cDNA yields were approximately 30ng/µl and 200ng/µl for the large and small molecules, respectively. Two µl of the large and 0.5µl of the small cDNA were ligated into the Stratagene UniZAP XR phage vector (Cat. # 237211) and packaged with Gigapack® III Gold packaging extract. There were 31100 primary plaques in the large cDNA library and 524,000 primary plaques in the small cDNA library. After amplification the titres of the large and small libraries were 8.7×10^9 pfu/ml and 9.7×10^9 pfu/ml respectively.

[0085] Twenty plaques from each library were picked into 0.5ml SM buffer (0.1M NaCl, 8mM $MgSO_4$, 50mM TRIS.HCl pH 7.5, 0.01% gelatin) 1% chloroform and eluted from agarose plugs by vortexing. Phage insert sizes were examined by PCR using T7 primers (5'TAA TAC GAC TCA CTA TAG 3') and T3 (5'AAT TAA CCC TCA CTA AAG 3'). Each 100µl reaction comprised 2µl eluted phage, 2µl 10mM dNTPs, 2µl of each primer (from stocks of 0.5µg/ml), 10µl 10X REDTaq (Sigma) PCR reaction buffer (100mM Tris-HCl pH 8.3, 500mM KCl, 11mM $MgCl_2$, 0.1% gelatin), 3µl REDTaq (Sigma) DNA polymerase (1 unit/µl in 20mM Tris-HCl, pH 8.0, 100mM KCl, 0.1mM EDTA, 1mM DTT, 0.5% Tween 20, 0.5% Igepal CA-630, inert dye, 50% glycerol) and 79µl ddH_2O. Thermal cycling (Hybaid Touchdown thermal cycler) parameters were 1X 94°C 4min, 30X 94°C 1min, 48.5°C 45s, 72°C 90 s, and 1X 72°C 5min. Agarose gel electrophoresis of the PCR products showed that large library inserts were $\geq$1600 base pairs and small library inserts $\leq$1600 base pairs.

Cloning cDNA of EV048

[0086] The N-terminal sequence determined for the HPLC fraction collected at 14.51min (designated EV048) from *H. bimaculata* SGE was used to design three degenerate primers (HF1, HF2, HF3) for use with the T7 primer (which binds to the UniZAP XR

vector), to amplify the cDNA encoding the peptide. The sequences of the primers were HF1 5'GAY GAR TGY CCN MGN ATN TG 3', HF2 5'GAR TGY CCN MGN ATN TGY AC 3', and HF3 5'ACN TTY GGN AAY CAR TG 3' (where Y = C or T, R = G or A, N = A or C or G or T, and M = A or C). Each 100µl reaction comprised 3µl large or small library, 3µl 10mM dNTPs, 2µl T7 and 4µl HF1 or HF2 or HF3 (from stocks of 0.5µg/ml), 10µl 10X REDTaq PCR reaction buffer, 3µl REDTaq DNA polymerase and 75µl dH_2O. Thermal cycling parameters were 1X 94°C 4min, 30X 94°C 1min, 48.5°C 45s, 72°C 90s, and 1X 72°C 5min.

[0087] Agarose gel electrophoresis revealed a range of PCR products. Five of these products were purified using a Qiaex II gel extraction kit (Qiagen) and sequenced with an ABI PRISM™ dye terminator cycle sequencing ready reaction kit and ABI sequencer (Perkin Elmer). Conceptual translation of one of the PCR products, derived from the small cDNA library using primer HF2 with T7, revealed an exact match with the N-terminal sequence of EV048. The sequence extended beyond the stop codon of the cDNA encoding the peptide. A reverse primer (HR1 5'AAT ACA ACA TAT TCA AGT GG 3') matching the region beyond the stop codon was used with the T3 primer (which binds to the UniZAP XR vector) to obtain the 5' end of the cDNA. The PCR product was cloned into the pGEM®-T Easy vector (Promega) then sequenced revealing a full-length cDNA encoding EV048.

Sequence analysis

[0088] Analyses were carried out using the GCG suite of programs (Wisconsin Package Version 10.1, Genetics Computer Group (GCG), Madison, Wisc.) and also the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (http://expasy.hcuge.ch/).

BAC-BAC® Baculovirus expression and purification of EV048

[0089] The HR1/T3 PCR product cloned into pGEM®-T Easy was amplified using primer HF6 (5' GTA *CGG ATC C*AT GAA ATT TGC CTT GTT CAG T 3') which matches the signal sequence of EV048 and has a *Bam HI* restriction enzyme site (italic), and primer HR3 (5' CAT G*CT GCA* **GTT** AGT GAT GGT GAT GGT GAT GAC CCT TGC ACT CGC CAT CATG 3') which matches the sequence encoding the carboxy-

terminal end of the protein and includes a codon for a glycine followed by six histidine residues (underlined) then a stop codon (bold) and *PstI* restriction enzyme site (italic). The 100µl reaction comprised 1ng HR1/T3 PCR product in pGEM®-T Easy in a volume of 1µl, 2.5µl 10mM dNTPs, 2µl HF6 and 2µl HR1 or HF2 (from stocks of 0.5µg/ml), 10µl 10X REDTaq PCR reaction buffer, 3µl REDTaq DNA polymerase and 77.5µl dH_2O. Thermal cycling parameters were 1X 94°C 4min, 20X 94°C 1min, 48.5°C 45s, 72°C 90s, and 1X 72°C 5min. The PCR product was gel purified, digested with *Bam HI* and *Pst I* and inserted into *Bam HI* and *Pst I* cut pFastBac1 plasmid (Gibco-BRL®). The sequence of the construct was verified by sequencing with primers PFBR (5' GAT TAT GAT CCT CTA GTA C 3') and PFBF (5' TAT TCC GGA TTA TTC ATA CC 3') which match pFastBac1 either side of the multiple cloning site of the plasmid. Transformation of the DH10α bacteria carrying the baculovirus-DNA, purification of the baculovirus DNA and generation of high titre baculovirus stock were performed in accordance with the instructions accompanying the BAC-BAC® baculovirus expression system (Gibco-BRL®).

[0090] For expression, *Sf*9 cells grown in Sf-900 II serum free medium (Gibco-BRL®) to a cell density of 1 x 10^6 cells/ml were infected at a multiplicity of infection of 5 and grown for a further 60 h. For purification, the cultures were centrifuged in a JA-20 rotor at 3000RPM for 10min and the supernatant was poured into beakers kept on ice. Polyethylene glycol MW 3350 was added to 30% (w/v) with stirring and the mixture was stirred for one hour. The mixture was centrifuged in a JA-20 rotor at 5000RPM for 20min and the protein pellet was resuspended in 20ml binding buffer (50mM Na_2HPO_4/NaH_2PO_4 pH 8, 500mM NaCl, 10% glycerol) per gram of wet paste. After addition of 500µl per gram of wet paste TALON Metal Affinity Resin (Clontech) the resuspended pellet was rocked for 1 hour on ice. The resin-binding buffer mix was brought onto a disposable 1ml purification column (Invitrogen). The resin was then washed with 10 volumes 10mM TRIS.HCl pH 8 and then with 15 volumes of wash buffer (50mM Na_2HPO_4/NaH_2PO_4 pH 6.5, 500mM NaCl, 10% glycerol). Bound proteins were eluted with 6 volumes 100mM NaH_2PO_4, 250mM imidazole and concentrated using Centricon 3 centrifugal filter devices (Amicon) spun in a JA-12 rotor at 5000RPM for 4 hours. Peptide EV048 was then purified further by cation exchange

chromatography. The buffer flow rate was 1ml/min for the washing and loading steps. An SP Sepharose cation exchange column (Pharmacia) was washed with 10 column volumes of running buffer (50mM Na_2HPO_4/NaH_2PO_4 pH 6.8) and the concentrated protein was diluted 200-fold in running buffer then passed over the column. After washing with a further 10 column volumes of running buffer, proteins were eluted using a 30 min 0 – 0.75M NaCl gradient at a flow rate of 0.5ml/min. Peptide EV048 eluted as a single peak which was concentrated and visualised on a 4-12% Bis-Tris polyacrlamide gel (Novex-Invitrogen) stained with GelCode Blue (Sigma).

Effect of crude Hybomitra bimaculata *salivary gland extract (SGE) on sarcolemmal Na, K-ATPase*

[0091] Cardiac sarcolemma was prepared from samples of the hearts of Wistar Kyoto rats by the hypotonic shock-NaI treatment method previously described (Vrbjar *et al* 1984). The protein content was assayed by the procedure of Lowry (Lowry *et al* 1951) using bovine serum albumin as a standard.

[0092] The substrate kinetics of Na,K-ATPase were estimated by measuring the splitting of ATP by 30-50 μg sarcolemmal proteins at 37°C in the presence of increasing concentrations of ATP in the range 0.08-4.0 mmol/l in a total volume of 0.5 ml of medium containing 50 mmol/l imidazole (pH 7.4), 4 mmol/l $MgCl_2$, 10 mmol/l KCl and 100 mmol/l NaCl. After 15 minutes of preincubation in the substrate free medium, the reaction was started by addition of ATP and 15 minutes later it was terminated by the addition of 1 ml of 12% solution of trichloroacetic acid. Verification of the time dependence of ATP-hydrolysis showed that for up to 20 minutes the ATP splitting was linear in the whole ATP concentration range applied. The inorganic phosphorous liberated was determined according to Taussky and Shorr (1953). In order to establish the Na,K-ATPase activity, the ATP hydrolysis that occurred in the presence of Mg^{2+} only was subtracted. From each sarcolemmal preparation three individual K_m and V_{max} values were obtained.

[0093] Crude salivary gland extracts (SGE) was obtained from *Hybomitra bimaculata.* The influence of this extract on the function of Na,K-ATPase was tested *in vitro* by addition of 0-10 μg of SGE to 30 μg of sarcolemmal proteins.

[0094] The kinetic parameters were evaluated by direct nonlinear regression of the data obtained. All results were expressed as mean ± SEM. The significance of differences between individual groups was determined by one way Bonferroni test (Sahai & Ageel, 2000), a value of $p<0.05$ being regarded as significant.

Isolated heart preparation and perfusion technique

[0095] Rat hearts were rapidly excised, placed in ice-cold perfusion buffer, cannulated via the aorta and perfused in the Langendorff mode at a constant perfusion pressure of 70 mm Hg and at 37°C. Perfusion solution was a Krebs Henseleit buffer gassed with 95% O_2 and 5% CO_2 (pH 7.4) containing (in mM): NaCl 118.0, KCl 4.7, $MgSO_4$ 1.66, $CaCl_2$ 2.52, $NaHCO_3$ 24.88, KH_2PO_4 1.18 and glucose 5.55. The solution was filtered through a 5 μm porosity filter (millipore).

[0096] An epicardial electrogram (EG) was registered by means of two stainless steel electrodes attached to the apex of the heart and the aortic cannula and continuously recorded (Miograph ELEMA-Siemens, Solna, Sweden). Heart rate was calculated from the EG.

[0097] Coronary flow was measured by a timed (10 s interval) collection of coronary effluent which was weighed on an electronic balance (AND HF 200 G, A&D Company Limited). Left ventricular pressure was measured by means of a latex water-filled balloon inserted into the left ventricle via the left atrium (adjusted to obtain end-diastolic pressure of 5-10 mm Hg) and connected to a pressure transducer (P23 Db Pressure Transducer, Gould Statham Instruments, Inc.)

[0098] Crude SGE of *Hybomitra bimaculata* was investigated. SGE from 8-20 salivary glands made up to 200 μl was injected through a syringe directly into the aortic cannula

with continuous measurement of coronary flow (CF), left ventricular pressure (LVP) and EG. Changes in CF, LVP and heart rate (HR) were evaluated.

Isolated rat femoral artery preparation

[0099] Wistar rats 12 weeks old of both sexes were used. After they were killed humanely, two segments (each approx. 10 mm in length) of femoral artery were isolated and placed in a Krebs-Ringer bicarbonate solution comprising 118 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4.7H_2O$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1 mM EDTA, 1.1 mM ascorbic acid, and 11 mM glucose. The endothelium was removed from one half of each segment preparation by gently rubbing the intimal surface. The vessel segments were cleaned of adherent connective tissue and cut into 3 mm ring segments. Two stainless-steel wires were passed through the lumen taking care not to damage the endothelium. The rings were mounted on a myograph capable of measuring the isometric wall tension and placed within a bath containing Krebs-Ringer solution through which was bubbled 95% O_2 and 5% CO_2, maintained at a temperature of 37°C and pH 7.4. The effectiveness of endothelium removal was demonstrated by failure of acetylcholine ($5x10^{-6}$ mol/l) to relax a contraction induced by phenylephrine ($5x10^{-6}$ mol/l). The plateau of the contractile response induced by phenylephrine ($5x10^{-6}$ mol/l) was taken as a measure of 100% contraction.

Effects of SGE on isolated rat ventricular myocytes

[0100] Rat ventricular myocytes were isolated by enzymatic digestion, layered in a glass perfusion chamber mounted on the stage of an inverted microscope, and perfused with a salt solution containing (mM): NaCl 140, KCl 54, $CaCl_2$ 1.0, $MgCl_2$ 1.0, glucose 10.0 and HEPES 10.0 (pH 7.35 adjusted with NaOH). The cells were field stimulated to contract at 0.5 Hz with 1.0 ms square wave pulses of supra-threshold voltage. The decrease in cell length during each contraction was measured with an edge detection system using a photodiode array. The temperature of the superfusate was 24±2°C. Data were digitised and acquired on a personal computer via an A/D converter using VCLAMP software (CED, Cambridge, UK).

[0101] To measure action potential and membrane currents, an Axoclamp 200 amplifier (Axon Instruments, Inc., Burlingame, Calif., USA) was used in whole-cell voltage clamp

or current clamp mode to record ionic currents and action potentials. Patch pipettes were pulled from filamented borosilicate capillary glass (GC150TF; Clark Electromedical Instruments, UK) on a microprocessor-based three-stage puller (Mecanex BB-CH-PC, Basel, Switzerland). The pipettes had resistances of 2-4 MΩ after filling with internal solution.

[0102] For action potential recordings, the pipettes were filled with a solution containing (in mM): KCl 140, $MgCl_2$ 1.0, Mg-ATP 5.0, Na_2-phosphocreatine 5.0, HEPES 5.0 (pH 7.2 adjusted with KOH). The external solution for these experiments was (mM): NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.0, glucose 10.0 and HEPES 5.0 (pH 7.4 adjusted with NaOH). Action potentials were elicited by injection of short current pulses.

[0103] To isolate whole-cell I_{Ca} the pipette was filled with a solution containing (mM): CsCl 120, $MgCl_2$ 1.0, Mg-ATP 5.0, Na_2-phosphocreatine 5.0, EGTA 10.0 and HEPES 5.0 (pH 7.2 adjusted with CsOH). Cells were superperfused with a solution containing (mM): tetraethylammonium-Cl 140, $CaCl_2$ 1.8, $MgCl_2$ 1.0, glucose 10.0, HEPES 5.0, and 4-aminopyridine 3.0 (at pH 7.4 adjusted with tetraethylammonium hydroxide). I_{Ca} was elicited by applying 100 ms depolarising voltage pulses in 5 mV steps from a holding potential of –45 mV. To measure whole-cell Current $_{\text{transient outward}}$ (I_{to}), the pipette was filled with a solution containing (mM): KCl 145, $MgCl_2$ 3.0, Mg-ATP 5.0, Na_2-phosphocreatine 5.0, EGTA 5.0 and HEPES 5.0 (pH 7.2 adjusted with KOH). Cells were superperfused with a solution containing (mM): choline-Cl 145, KCl 5.4, $CaCl_2$ 0.5, $MgCl_2$ 0.5, glucose 5.5, $CoCl_2$ 2.0 and HEPES 5.0 (pH 7.4 adjusted with KOH). I_{to} was elicited by applying 100 ms depolarising voltage pulses in 10 mV steps to +70 mV from a holding potential of –80 mV. In these experiments, the interval between pulses was 5 seconds and the superfusate temperature was 35±1°C.

[0104] The contractile and electrophysiological effects of the 56 amino acid recombinant peptide from *Hybomitra bimaculata* (EV048) was tested on single cardiomyocytes isolated from rat ventricles.

Results

Horse fly collection

[0105] In Slovakia, 63 horse fly species have been recorded. Approximately one third of these species are very common, one third are common in appropriate biotopes, and the remaining species are rare. In this collection, 16 horse fly species were present of which *Hybomitra bimaculata* was the second most abundant species, a total of 1300 individuals being collected.

Effect on sarcolemmal Na,K-ATPase

[0106] The influence of salivary gland extract (SGE) from *Hybomitra bimaculata* on the function of Na,K-ATPase was tested by the addition of various amounts of SGE to 30 µg of sarcolemmal proteins. Na,K-ATPase, an enzyme involved in the active translocation of Na^+ and K^+ ions across cell membranes causes the potassium dependent relaxation or so-called hyperpolarisation. For this purpose the enzyme utilises the energy derived from hydrolysis of ATP. Therefore, in the present study attention was focused on the influence of SGE on the ATP-binding properties of the enzyme by investigating its behaviour in the presence of increasing concentrations of ATP.

[0107] The result of this experiment is shown in Figures 2 & 3. The result clearly showed that SGE from *Hybomitra bimaculata* contains at least one compound which at lower concentrations stimulates Na,K-ATPase but at the highest concentration tested (6.5 µg) has an inhibitory effect. This biphasic reaction suggests that at lower concentrations the salivary gland extract is able to increase the hyperpolarisation of muscle cells but at higher concentrations it has the reverse effect.

Vasodilating activity of rat femoral artery induced by SGE

[0108] The relaxing responses of rat femoral artery induced by SGE from *Hybomitra bimaculata* was examined. Using rat femoral artery with intact endothelium, the application of 50 µl SGE (equivalent to ½ salivary gland) from *Hybomitra bimaculata* induced 119% relaxation (Figure 4).

[0109] Removal of endothelium did not decrease the vasodilating responses induced by SGE. In fact, SGE from *Hybomitra bimaculata* induced 39% ($p<0.05$) greater relaxation of the artery than with the endothelium intact (Figure 4). In view of this finding the effects of fractions obtained by HPLC on endothelium-denuded rings was investigated.

[0110] The vasodilating responses of endothelium-denuded arterial rings induced by protein HPLC fractions of salivary glands from *Hybomitra bimaculata* obtained in the retention time range 10-28 min were compared. The maximum vasodilating responses were induced by a fraction with the retention time 13.77 min (47% relaxation; Figure 5).

Effect of Hybomitra bimaculata SGE on $NaHPO_4$ perfused isolated rat heart

[0111] The underlying principle of the Langendorff model of perfusion of isolated rat heart (Langendorff, 1895) is to force blood, or any other oxygenated fluid appropriate to maintain cardiac activity, towards the heart through a cannula inserted into the ascending aorta. Retrograde perfusion closes the aortic valves (mimicking the *in situ* heart during diastole) and the perfusate is displaced through the coronary arteries. After passing through the coronary vascular system, the perfusate flows through the coronary sinus and the opened right atrium, respectively. The cardiac cavities remain basically empty throughout the experiment. The primary reason for the investigation of a substance with an unknown effect in an isolated organ is the very independence of this isolated organ from nervous and humoral regulation as well as from the substrate supply by the complete organism.

[0112] SGE from *Hybomitra bimaculata* that had displayed the maximal vasodilatory activities in rat femoral artery rings was selected for testing in the isolated perfused rat heart model (Langendorf constant pressure model). In this model, the *Hybomitra bimaculata* SGE increased coronary flow and left ventricular contractility, the most potent being SGE at the 100 µl dose. The results of this experiment are shown in Table 1 and Figure 6.

[0113] Table 1: Effect of salivary gland extract from Hybomitra bimaculata on the isolated perfused rat heart.

Dose SGE (µl)	Coronary flow	Left Ventricular Contractility	Change in heart rate
50	+39%	+20%	0
100	+50%	+42.8%	-10%
150	+42%	+40%	+5%

Purification and identification of active fractions from Hybomitra bimaculata *SGE*

[0114] For the purification and identification of active fractions, salivary glands of *Hybomitra bimaculata* were used. Figure 7 demonstrates the RP-HPLC chromatogram obtained from SGE of 475 pairs of salivary glands. A vasorelaxation activity of 47% was found in the peak with a retention time of 13.77 min; 45% relaxation was measured in the peak with a retention time of 16.28 min. Less activity (30%) was obtained with a peak of retention time 9.51 min, and 15% with a peak of retention time 22.47 min.

Amino acid analysis and sequencing of Hybomitra bimaculata *HPLC fractions*

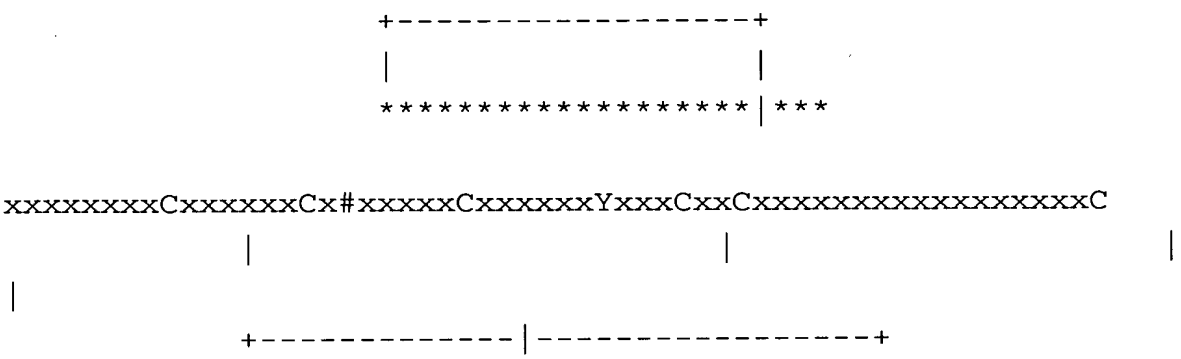
[0115] The HPLC fraction of retention time 14.51 min, which was derived from the 13.77 min peak (Figure 7), was subjected to N-terminal Edman degradation and yielded a partial sequence of 47 amino acid residues (Figure 8), designated EV048.

Primary structure of the cDNA encoding EV048

[0116] The full length cDNA for EV048 encodes a peptide of 76 amino acids (Figure 9a). This includes a 20 amino acid putative signal peptide that is probably cleaved at VAA – DEC to generate the mature N-terminus (Figure 8). The complete peptide has a predicted molecular weight of 8282.4 Da and a theoretical pI of 8.27. The 56 amino acid mature peptide has a predicted molecular weight of 6146.7Da and a theoretical pI of 7.78. An N-linked glycosylation site is predicted at the asparagine residue at position 26 in the mature peptide; there are no predicted O-linked sites. The sequence of the mature peptide has similarity with Kazal-type protease inhibitors (Figure 10), including homology with rhodniin I and II, the Kazal-type inhibitors from *Rhodnius prolixus*, another haematophagous insect species (Friedrich et al., 1993, van de Locht et al., 1995),

and with the protease inhibitor from the sea anemone *Anemonia sulcata* that is specific for elastases (Tschesche *et al.*, 1987).

[0117] The basic structure of a Kazal-type inhibitor is shown in the following schematic representation:



[0118] All six cysteines are conserved in EV048 although the spacing between cysteine residues within the consensus pattern is unusual C–x(7)– C– x(13)–F –x(3)– C– x(6)– C. This unusual pattern in EV048 is due to a sequence inserted between the third and fourth cysteines (PSGGRRS) that does not align with any other Kazal family member (Figure. 10). The second serine within the sequence has a high probability of phosphorylation but the rest of the seven amino acid sequence has no similarity to any other motifs in the PROSITE database. Homology modelling indicates that the insertion occurs within, or just before, the region of the peptide that forms the second β-sheet. The highly conserved tyrosine residue (shown in bold in Figure 10) is a phenylalanine in EV048, and the putative active site residue (indicated by # in bold in Figure 10) is an alanine.

Baculovirus expression of EV048

[0119] The expressed peptide is exported from the cell to the supernatant. Expression levels of EV048 in the supernatant were approximately 0.3μg per ml of Sf9 cells. The

expressed protein is approximately the size (7 kD) expected for the mature peptide (with glycine and 6 X HIS tag) and was purified to homogeneity (Figure 11) in two steps.

Activity of EV048 on isolated rat cardiomyocytes

[0120] EV048, the recombinant peptide, derived from saliva of *Hybomitra bimaculata* (0.2 and 0.4 µg/ml) exhibited positive inotropism and prolongation of the action potential for the duration of exposure. These effects persisted for up to 16.5 minutes following washout. Spontaneous contractile activity was not observed at any time with this molecule. The results of a series of three experiments examining the effect of EV048 on the action potential of rat cardiomyocytes are shown in Figure 12.

Effect of EV048 on the isolated perfused rat heart

EV048 was tested in the isolated perfused rat heart model. This resulted in a transient increase in coronary blood flow with no alteration of heart rate or rhythm (Figures 13a and b).

Discussion

Vasodilatory effects of Hybomitra bimaculata *salivary gland extract (SGE)*

[0121] SGE from *Hybomitra bimaculata* induced relaxation of rat femoral artery exceeding 50%. Similar magnitude of arterial relaxation has been induced by SGE from several species of haematophagous insects including mosquitoes (Champagne and Ribeiro, 1994), black flies (Cupp et al., 1994), sand flies (Lerner and Shoemaker, 1992), ticks (Kemp et al., 1983) and triatomine bugs (Ribeiro et al., 1990, 1993). However the mode of action of the peptidic vasodilators found in the other species varies. None are ion channel modulators.

[0122] Tachykinins elicit release of nitric oxide following binding of the peptide to endothelial cell tachykinin receptors. Such binding induces endothelium-dependent vasorelaxation (Champagne and Ribeiro, 1994). By contrast, protein vasodilators such as nitrophorins are able to bind and release nitric oxide. Delivering NO to the host vessel induces direct relaxation of smooth muscle by increasing intracellular cGMP levels (Champagne, 1994; Weichsel *et al.*, 1998). Other vasodilators like maxadilan increase the intracellular level of cAMP within smooth muscle cells leading to relaxation

(Grevelink *et al.*, 1995). Both vasodilating mechanisms are endothelium-independent. It is proposed that, unlike vasodilators found in other haematohagous arthropod species, EV048 does not act either as a tachykinin or as a NO donor.

[0123] Data presented here support endothelium-independent relaxation of rat femoral artery induced by *Hybomitra bimaculata* SGE. Indeed, SGE from this species induced higher levels of relaxation of the artery after endothelium removal. SGE from this horsefly species appears to act directly on smooth muscle cells, presumably by calcium channel blocking, and promote vasorelaxation without endothelium mediation, the endothelium representing, if anything, a barrier rather than part of the active process.

Effects on sarcolemmal Na,K-ATPase:

[0124] The results clearly showed that the SGE from *Hybomitra bimaculata* contains at least one compound which, at low concentration, stimulates Na,K-ATPase but at higher concentration inhibits the enzyme. When 3µg of SGE were applied it induced a significant stimulation of the Na,K-ATPase. Increasing the amount of SGE to 6.5 µg induced an inhibition of the Na,K-ATPase. This phenomenon may be fundamental to understanding the vasodilating activity of this SGE on the rat femoral artery, whilst also explaining the positive inotropism of the SGE from *Hybomitra bimaculata* in the isolated rat heart and of the recombinant molecule EV048 in isolated rat cardiomyocytes. Inhibition of Na,K-ATPase, as was found at higher concentrations of SGE, has long been known to be associated with positive inotropism as is the case with other inhibitors of such as ouabain (Allen *et al.* 1975) and vanadium (Schmitz *et al.* 1982).

[0125] Whilst the inhibitory effect on Na,K-ATPase of SGE from *Hybomitra bimaculata* may explain the positive inotropism of the same SGE in the isolated rat heart, it is difficult to explain the substantial relaxation of femoral artery smooth muscle when exposed to *Hybomitra bimaculata* SGE by the same mechanism and this, together with the increase in diastolic volume and the increase in coronary blood flow in the rat heart model, are more easily explained by calcium channel inhibition. However, the fact that stripping the vascular endothelium appears to enhance the degree of smooth muscle relaxation could also suggest the involvement of NO.

Cardioactive effects of the crude Hybomitra bimaculata *SGE and a recombinant peptide molecule, EV048, on the isolated perfused rat heart*

[0126] The initial results of testing crude extracts of salivary glands from *Hybomitra bimaculata* in an isolated perfused rat heart model resulted in both increased coronary blood flow and left ventricular contractility. Investigation of EV048 in this model suggested that it has a positively inotropic effect, although limited availability of material meant that this effect was transient. No negative inotropism was seen with either the SGE or the recombinant molecule and there were no alterations in heart rate or rhythm. This combination of features in a potent vasodilator such as EV048 is unusual, if not unique, and endows it with substantial therapeutic potential.

Effects of a recombinant peptide, EV048, on isolated rat cardiomyocytes

[0127] EV048 prolonged the action potential and showed positive inotropism in isolated rat cardiomyocytes without provoking spontaneous contractile activity. This combination of properties, together with the substantial increase in coronary blood flow caused by *Hybomitra bimaculata* SGE in the isolated perfused rat heart, is considered to give EV048 potential value as a therapeutic agent in situations where increased ventricular output is desirable without the risk of inducing arrhythmias. Such clinical situations might include haemorrhagic, cardiogenic and septic shock, intractable angina and heart failure following coronary thrombosis. The duration of action after removal of the active agent by washout (> 16 minutes) is also considered to increase its potential value as a therapeutic agent. Prolongation of the action potential without apparently inducing spontaneous oscillations may also make it a useful anti-arrhythmic agent.

Kazal-type protein from Hybomitra bimaculata

[0128] Following protein fractionation of SGE from *Hybomitra bimaculata* by HPLC, the maximal vasodilating responses of the artery were induced by fractions with a retention time of 9.51, 13.77, 16.28, and 22.47 min (Figure 6).

[0129] The amino acid analysis of the fraction with the maximum-inducing vasorelaxation (retention time 13.77 min), and subsequent analysis of the derived cDNA, indicated that the molecule designated EV048 is closely related to Kazal-type proteins. The Kazal family of proteins includes a variety of protease inhibitors including

pancreatic secretory trypsin inhibitor (Greene and Giordano, 1969), avian ovomucoid (Laskowski *et al*, 1987), acrosin inhibitor (Williamson *et al*, 1984) and elastase inhibitor (Tschesche *et al*. 1987). Kazal inhibitors contain between 1 and 9 Kazal-type inhibitor repeats. Kazal protease inhibitors that inhibit trypsin-like proteinases have basic residues (R, K or H) at their active (or P1) site whereas those that inhibit chymotrypsin-like proteases have large hydrophobic residues at the P1 position. The putative active site residue of EV048 is the small hydrophobic amino acid alanine. This residue is not present in any other Kazal proteins known to inhibit proteases. However, the active site of EV048 appears similar to a modelled sequence predicted to have very tight binding to porcine pancreatic elastase (Lu et al, 2001).

[0130] The extra sequence (PSGGRRS) inserted between the third and fourth cysteine residues of EV048 may well play a role in the vasodilating properties of the peptide. Homology modelling suggests that the additional amino acids exist at an exposed location and may permit interaction with a target molecule. Initial studies with the recombinant molecule presented here have demonstrated biological activity consistent with the effects observed with crude SGE from *Hybomitra bimaculata.*

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